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Ecteinascidin 743 Interferes with the Activity of EWS-FLI1 in Ewing Sarcoma Cells[1,2]

Patrick J. Grohar*, Laurie B. Griffin*, Choh Yeung*, Qing-Rong Chen†, Yves Pommier‡, Chand Khanna§, Javed Khan† and Lee J. Helman*

*Molecular Oncology Section, Pediatric Oncology Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA; †Oncogenomics Section, Pediatric Oncology Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA; ‡Laboratory of Molecular Pharmacology, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA; §Tumor and Metastasis Biology Section, Pediatric Oncology Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA

Abstract

ET-743 (trabectedin; Yondelis) is approved in Europe for the treatment of soft tissue sarcomas. Emerging phase 1 and 2 clinical data have shown high response rates in myxoid liposarcoma in part owing to the inhibition of the FUS-CHOP transcription factor. In this report, we show that modulation of specific oncogenic transcription factors by ET-743 may extend to other tumor types. We demonstrate that, among a panel of pediatric sarcomas, Ewing sarcoma family of tumors (ESFTs) cell lines bearing the EWS-FLI1 transcription factor are the most sensitive to treatment with ET-743 compared with osteosarcoma, rhabdomyosarcoma, and synovial sarcoma. We show that ET-743 reverses a gene signature of induced downstream targets of EWS-FLI1 in two different ESFT cell lines ($P = .001$). In addition, ET-743 directly suppresses the promoter activity of a known EWS-FLI1 downstream target NR0B1 luciferase reporter construct without changing the activity of a constitutively active control in ESFT cells. Furthermore, the effect is specific to EWS-FLI1, as forced expression of EWS-FLI1 in a cell type that normally lacks this fusion protein, HT1080 cells, induces the same NR0B1 promoter, but this activation is completely blocked by ET-743 treatment. Finally, we used gene set enrichment analysis to confirm that other mechanisms of ET-743 are active in ESFT cells. These results suggest a particular role for ET-743 in the treatment of translocation-positive tumors. In addition, the modulation of EWS-FLI1 makes it a novel targeting agent for ESFT and suggests that further development of this compound for the treatment of ESFT is warranted.

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Abbreviations: ET-743, trabectedin; Yondelis; ESFT, Ewing sarcoma family of tumors; TCR, transcription-coupled nucleotide excision repair; GSEA, gene set enrichment analysis

Address all correspondence to: Patrick J. Grohar, MD, PhD, Pediatric Oncology Branch, Bldg 10, Rm CRC-1W-3750, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892-1928. E-mail: groharp@mail.nih.gov

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Introduction

The Ewing sarcoma family of tumors (ESFTs) is the second most common malignant bone tumor of childhood [1]. The hallmark of the disease is the characteristic chromosomal translocation involving the EWS gene and one of the members of the *ets* family of transcription factors, most commonly FLI1 [2]. This translocation fuses the DNA binding domain of FLI1 with the transactivating domain of EWS, leading to a dysregulated transcription factor that alters the expression of more than 500 genes [2–6].

Downstream targets of EWS-FLI1 have been shown to mediate oncogenic transformation [7], regulate the cell cycle [8,9], and participate in the evasion of apoptosis [10,11] and cellular senescence [12]. Furthermore, a number of groups have shown that decreasing the expression of EWS-FLI1 by antisense DNA or small interfering RNA methods decreases the proliferation of ESFT cells *in vitro* and decreases tumorigenicity in orthotopic mouse models (reviewed in Maksimenko and Malvy [13]). Therefore, agents that modulate EWS-FLI1 would be of clinical interest.

ET-743 (trabectedin; Yondelis) is a natural product isolated from the sea squirt *Ecteinascidia turbinata* [14,15]. This drug has shown cytotoxicity in a variety of histologic cell types, and recent clinical studies have led to approval in Europe for the treatment of soft tissue sarcomas. Specifically, phase 1 and 2 data show high response rates in myxoid liposarcoma, which is known to have the t(12;16)(q13;p11) chromosomal translocation that generates the FUS-CHOP fusion protein [16–22].

Recently, it has been shown that ET-743 blocks DNA binding of the oncogenic transcription factor FUS-CHOP and reverses the transcriptional program in myxoid liposarcoma. By reversing the genetic program created by this transcription factor, ET-743 promotes differentiation and reverses the oncogenic phenotype in these cells [23].

Other than transcriptional interference, the mechanism of action of ET-743 is complex and not completely understood. The compound is known to bind and alkylate DNA at the N2 position of guanine. It is known from *in vitro* work that this binding occurs in the minor groove, spans approximately 3 to 5 bp and is most efficient with CGG sequences [24]. Additional favorable binding sequences are TGG, AGC, or GGC [24,25]. Once bound, this reversible covalent adduct bends DNA toward the major groove [26], interferes directly with activated transcription [27,28], poisons the transcription-coupled nucleotide excision repair (TCR) complex [29–31], promotes degradation of RNA polymerase II [32], and generates DNA double-strand breaks [33,34].

Although a considerable amount of mechanistic work has been done with ET-743, it is not known what determines preferential sensitivity to the drug in the clinic. Furthermore, it is not known if other specific oncogenic transcription factors could be targeted by ET-743 or if this targeting could provide another mechanism of cytotoxicity that is additive with other known mechanisms of action.

In this report, we hypothesized that ET-743 may interfere with the transcriptional program of EWS-FLI1 at the promoter level similar to FUS-CHOP in myxoid liposarcoma. We based this hypothesis on several factors including the fact that FUS and EWS are family members. We show that ET-743 does in fact reverse a gene signature of EWS-FLI1 and heightened sensitivity is observed *in vitro* relative to other pediatric sarcomas that lack the EWS-FLI1 transcription factor. In addition, we demonstrate specific suppression of a downstream target promoter both in a native and artificial context. Finally, we show preservation of other previously described mechanisms of action in ESFT and the potent induction of apoptosis at nanomolar concentrations in ESFT cells.

Materials and Methods

Cell Culture

ESFT cells TC32, TC71, and 5838 have been described and characterized previously [35]. The presence of the EWS-FLI1 transcription factor was routinely confirmed by reverse transcription–polymerase chain reaction (PCR). The rhabdomyosarcoma cell line RD and the osteosarcoma cell lines HOS, SaOS, and U2OS were obtained from American Type Culture Collection (Rockville, MD). The RH30 and EW8 cells were gifts from Dr P. Houghton. SCS991 synovial sarcoma was patient-derived, and the SYT-SSX2 translocation was confirmed by reverse transcription–PCR. Cells were cultured in RPMI-1640 (Gibco, Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (Sigma, St Louis, MO) and supplemented with penicillin/streptomycin and L-glutamine to a final concentration of 100 U/ml, 100 μg/ml, and 2 mM, respectively.

Cell Proliferation Assays

Cells were plated to a density of 3000 to 5000 cells per well and allowed to recover overnight. They were subsequently treated with ET-743 dissolved in 1% DMSO and diluted to the given concentration maintaining the final concentration of DMSO at less than 0.0001%. To measure the cell number, 20 μl of CellTiter 96 (Promega, Madison, WI) was added to a volume of 100 μl of medium and allowed to incubate at 37°C for 2 hours. The color change of the well was measured with a 96-well plate reader and plotted against a standard curve to determine the cell number. All treatments were done in replicates of three to six.

Luciferase Assays

The NR0B1 promoter was PCR-amplified from genomic DNA using 5′GGTACCGTCCTAGAACTTCCAGAAG3′ as the forward primer and 5′GCTAGCCTTCGCTCATAAGCATG-3′ as the reverse primer, containing the restriction sites *Kpn*I and *Nhe*I, respectively. The insert was cloned into the TOPO-TA shuttle vector (Invitrogen), subsequently digested using *Kpn*I and *Nhe*I restriction enzymes, and ligated into the PGL 4.18 vector (Promega). The plasmid was linearized with *Sal*I and cloned into TC32 cells using O-017 program and buffer R as per the Amaxa nucleofection protocol. Single-cell clones were grown under selective pressure using G418 at a final concentration of 0.5 mg/ml.

The CMV promoter was PCR-amplified from PGL 4.75 plasmid DNA (Promega) and cloned into PGL 4.18 using the In-Fusion system (Clontech, Mountain View, CA). Primers were designed with a 15-bp overlap using online software with the 5′ACCTAGCTCGCTAGCGGCTAACTGGCTCAATA 3′ forward primer and the 5′TATCCTCGAGGCTAGCCCACTGACTGCGTTAGCAAT 3′ reverse primer. After PCR amplification, the insert was combined with plasmid in a 2:1 ratio as per the manufacturer's instructions. The purified plasmid was nucleofected into TC32 cells, and single-cell clones were expanded as previously mentioned. All plasmids were sequenced to confirm the construct.

Luciferase assays were performed using Steady-Glo (Promega) prolonged signal luciferin. Cells were incubated with drug for the designated time. One hundred microliters of Steady-Glo was subsequently added to the cells. The cells were disrupted and allowed to equilibrate at room temperature for 5 to 10 minutes before measuring the bioluminescent signal on the Victor3 multilabel counter (Perkin Elmer, Waltham, MA).

Preparation of HT1080 Cell Lines That Express EWS-FLI1

EWS-FLI1 was cloned from Ewing cells and packaged into a lentiviral vector. The lentiviral particles of EWS-FLI1 and GFP were prepared by Betty Conde at the NCI Frederick and titered to a final density of at least 1×10^6 infectious units per milliliter. The viral supernatant was added to the HT1080 cells in the absence of antibiotics with a multiplicity of infection of at least 10 in the presence of 6 μg/ml polybreen. The cells were allowed to recover overnight and subsequently batch-selected under selective pressure with blasticidin at a final concentration of 10 μg/ml.

Preparation of HT1080 Reporter Cell Lines

The NR0B1 promoter luciferase construct (described previously) was cloned into the transgenic EWS-FLI1 expressing HT1080 cells using nucleofection with the Amaxa system as per the manufacturer's instructions. The CMV promoter construct (described previously) was also nucleofected using the identical conditions. The cells were subsequently batch selected using G418 at a final concentration of 0.5 mg/ml.

Western Blot Analysis

TC32 and TC71 cells were plated and allowed to recover overnight. The cells were treated with ET-743 at 10 and 1 nM for 12 hours and subsequently scraped into PBS. The cells were lysed using LDS lysis buffer (Sigma), and the protein was quantitated by using a BCA colorimetric assay (Pierce, Thermo-Scientific, Rockford, IL). After loading on a NuPage 4% to 12% gradient gel (Invitrogen), the lysates were transferred to nitrocellulose using the iBlot system (Invitrogen). The blots were probed with 2 μg/ml mouse anti-FLI1 (BD Pharmingen, San Diego, CA) and secondary (1:1000) antimouse (Cell Signaling, Danvers, MA). The poly (ADP-ribose) polymerase antibody (Cell Signaling) was used in a dilution of 1:1000. The bands were visualized on film using ECL detection reagent (Amersham, GE Healthcare, Little Chalfont, UK).

Microarray Analysis of Downstream Targets

TC32 and TC71 cells were plated in parallel and allowed to recover overnight. The cells were treated with ET-743 at a final concentration of 10 nM and allowed to incubate for 12 hours. The medium was aspirated, and the cells were washed with PBS. RNA was collected using TRIzol (Invitrogen) and the RNeasy kit (Qiagen, Germantown, MD) as per standard methods. RNA integrity was confirmed by using the bio-analyzer (Agilent, Santa Clara, CA) according to standard methods. Gene expression profiling was performed using the Affymetrix GeneChip Human Genome U133 Plus 2.0 microarray. RNA was *in vitro* transcribed, fragmented, hybridized, and stained using the appropriate Affymetrix GeneChip kits as per the manufacturer's instructions. The .CEL files were exported from Affymetrix GCOS software and normalized with RMAketch from Affymetrix Power Tools. The gene set enrichment analysis (GSEA) method (http://www.broad.mit.edu/gsea/) [36] was applied to investigate the enrichment of EWS-FLI downstream targets. GSEA analysis was completed with a weighted enrichment statistic, and genes were ranked using $\log_2$ ratio of expressions between ET-743–treated cells and control.

Results

ESFT Cells Are More Sensitive to ET-743 Treatment than Other Pediatric Sarcoma Cell Lines

To test our hypothesis that EWS-FLI1 inhibition by ET-743 adds an additional mechanism of toxicity specific to ESFT cells, we looked at the relative sensitivity of ESFT compared with other pediatric sarcoma cell lines that lack the EWS-FLI1 transcription factor. We treated a series of cell lines representing ESFT, osteosarcoma, embryonal rhabdomyosarcoma, alveolar rhabdomyosarcoma, and synovial sarcoma with ET-743. The ESFT cell lines were the most sensitive to 1 nM ET-743 at 48 hours, demonstrating greater than 50% inhibition of proliferation at this concentration (Figure 1*A*). In comparison, all osteosarcoma cell lines evaluated showed a less than 10% inhibition at the identical time and concentration.

Interestingly, 5838 was the least sensitive of the four ESFT cell lines tested. This cell line harbors the EWS-ERG translocation, whereas TC32, TC71, and EW8 all are characterized by the EWS-FLI1 translocation. It is known that all *ets* transcription factors share the same GGA(A/T) core binding domain [37], but it is not clear beyond the core sequence what additional sequence requirements exist.

It is also interesting to note that the alveolar rhabdomyosarcoma cell line RH30 was more sensitive than the embryonal cell line RD. RH30 cells contain the PAX3-FKHR translocation. FKHR belongs to the same winged helix turn helix family of DNA binding transcription factors as FLI1, although the binding motifs are different [3,38]. Taken together, the data demonstrated greater sensitivity of ESFT containing EWS-FLI1, all with subnanomolar half-maximal inhibitory concentration (IC_{50}) values, compared with other sarcoma cell lines (Figure 1*B*), and suggest that the antiproliferative effect may be connected to the EWS-FLI1 transcription factor.

ET-743 Induces Apoptosis and Decreases the Expression of Multiple EWS-FLI1 Downstream Targets

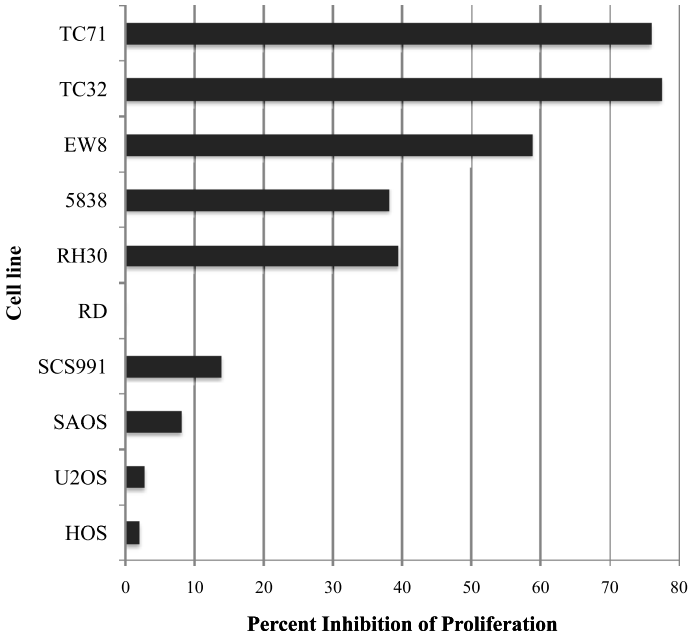
To investigate whether the heightened sensitivity of ESFT cells to ET-743 is related to suppression of the EWS-FLI1 transcriptional program, we looked at the effects of ET-743 on gene expression in ESFT cells in two different cell lines, TC32 and TC71. We used GSEA to determine whether suppression of the gene signature of EWS-FLI1 occurs with ET-743 treatment [36]. GSEA allows one to determine the placement of a set of genes on a second rank-ordered gene list. In this case, we treated cells with ET-743, evaluated the changes in gene expression using Affymetrix microarrays, and ranked the genes from the most induced with treatment to the most suppressed. We then looked for the placement of a list of published downstream targets that are induced by EWS-FLI1 on this list [5]. We found most genes clustered on the suppressed side of the gene list with an overall statistically significant suppression of the gene signature of EWS-FLI1 with ET-743 treatment ($P < .001$; Figure 2*A*). Of the 61 genes evaluated, 49 (80%) showed decreased expression. There was very little induction of genes that are suppressed by EWS-FLI1 (data not shown).

This effect could not be accounted for by nonspecific cytotoxicity because there were a large number of genes induced with treatment and no apoptosis observed at the 12-hour time point and 10-nM concentration tested as shown by Western blot (Figure 2*B*). Furthermore, if there were no relationship between treatment and this gene set, these genes would randomly distribute on the rank-ordered list and not generate a statistically significant effect. Finally, there was a potent induction of apoptosis as demonstrated by an increase in cleaved poly (ADP-ribose) polymerase that followed this suppression at 24 hours that would be expected with suppression of EWS-FLI1 (Figure 2*C*).

ET-743 Inhibits the Promoter Activity of the EWS-FLI1 Target Gene NR0B1

To further investigate whether the enhanced ET-743 effect in ESFT cells is due to the inhibition of EWS-FLI1 activity, we designed

A



B

Cell Line	Sarcoma type	IC50 (nM)
TC32	Ewing's Type 1	0.4
TC71	Ewing's Type 1	0.4
EW8	Ewing's Type 1	0.5
5838	Ewing's EWS-ERG	UTD
SCS991	Synovial Sarcoma	2
RH30	Alveolar Rhabdomyosarcoma	1.5
RD	Embryonal Rhabdomyosarcoma	4
HOS	Osteosarcoma	6
U2OS	Osteosarcoma	4
SAOS	Osteosarcoma	4

Figure 1. Ewing sarcoma cell lines that contain the EWS-FLI1 transcription factor are particularly sensitive to ET-743 treatment. (A) TC71, TC32, and EW8 show a 60% to 75% inhibition of proliferation relative to solvent control when treated with ET-743 at a concentration of 1 nM for 48 hours. All other sarcoma cell lines show a more modest inhibitory effect. (B) IC_{50} values for the various cell lines treated with ET-743. The 5838 cell line showed a plateau effect at approximately 53% and therefore the IC_{50} could not be determined (UTD).

a luciferase reporter construct that uses the promoter of a well-characterized EWS-FLI1 downstream target, NR0B1, to drive the expression of luciferase. The luciferase activity of these cells was measured relative to a constitutively active CMV-driven luciferase to control for nonspecific transcriptional inhibition or a cytotoxic reduction in luciferase expression.

Because knockdown of EWS-FLI1 is lethal to cells, the best time point to evaluate the luciferase reporter construct is at a time before cell death. Therefore, we evaluated the luciferase expression of the NR0B1 reporter and CMV control after 12 hours of exposure to ET-743. Treatment of TC32 cells containing the NR0B1 luciferase construct with ET-743 resulted in a marked dose-dependent inhibition of luciferase activity of between 50% and 80% at 12 hours (Figure 3*A*). There was very little inhibition observed in the CMV-driven luciferase at the same time and concentrations tested (Figure 3*A*). This inhibition occurred in the nanomolar range, and this suggests direct modulation of the EWS-FLI1 transcription factor by ET-743. It should be noted that this effect is not due to the nonspecific cytotoxicity (Figure 3*B*). Furthermore, prolonged exposure of ESFT cells to ET-743 demonstrates the same effect at concentrations closer to the IC_{50} when the data are expressed normalized to cell number (Figure 3, *C* and *D*).

ET-743 Blocks the Activation of the NR0B1 Promoter That Occurs with Forced Expression of EWS-FLI1 in HT1080 Cells

To determine whether the modulation of EWS-FLI1 is specific to this transcription factor, we expressed EWS-FLI1 in the non–EWS-FLI1–bearing HT1080 fibrosarcoma cell line and subsequently evaluated the effect of ET-743 treatment on the NR0B1 and CMV luciferase reporter constructs (Figure 4*A*). After confirming that transduction of the HA-tagged EWS-FLI1 in the HT1080 cells was stable by Western blot analysis (Figure 4*B*), we showed that EWS-FLI1 increases the activation of the NR0B1 promoter by 40% relative to the GFP transduction control ($P = .0008$; Figure 4*C*). Treatment of these cells with ET-743, however, completely blocks this activation at concentrations as low as 10 nM in a dose-dependent fashion ($P = .00005$; Figure 4*C*). The suppressive effect could not be accounted for by a decrease in cell number, as no statistically significant difference in viability was found between treatment and controls because the cells were once again evaluated at a 12-hour time point before an effect on viability occurs (data not shown). In contrast, the CMV promoter–driven luciferase demonstrated high-level expression typical of a constitutively active promoter construct, but no change in bioluminescence was observed relative to the GFP control with forced expression of EWS-FLI1 (Figure 4*D*). In addition, no interference with expression of luciferase was observed with ET-743 treatment in this constitutively active CMV-driven reporter construct (Figure 4*D*).

GSEA Confirms Previously Reported Mechanisms of Action of ET-743 and Demonstrates Induction of Genes Involved in Apoptosis

Next, we investigated similarities between the gene signature altered by ET-743 in ESFT cells and published curated gene sets. The Broad Institute maintains a database of more than 1890 microarray experiments (http://www.broad.mit.edu/gsea/) [36]. The analysis looks for similarities in expression between the target gene set (ET-743 treatment of ESFT cells) and any of the 1890 gene sets and reports the size (number of genes that overlap) as well as the statistical similarity both as a *P* value and a false discovery rate (FDR; Tables W1 and W2). The top 11 gene sets identified after sorting the list for size and maintaining $P < .001$ and FDR < 0.03 are shown in Table 1.

A number of gene sets that represent DNA damage were identified using these criteria. Most notably, data sets generated by treating normal human epidermal keratinocyte cells with UVB or either UVB or

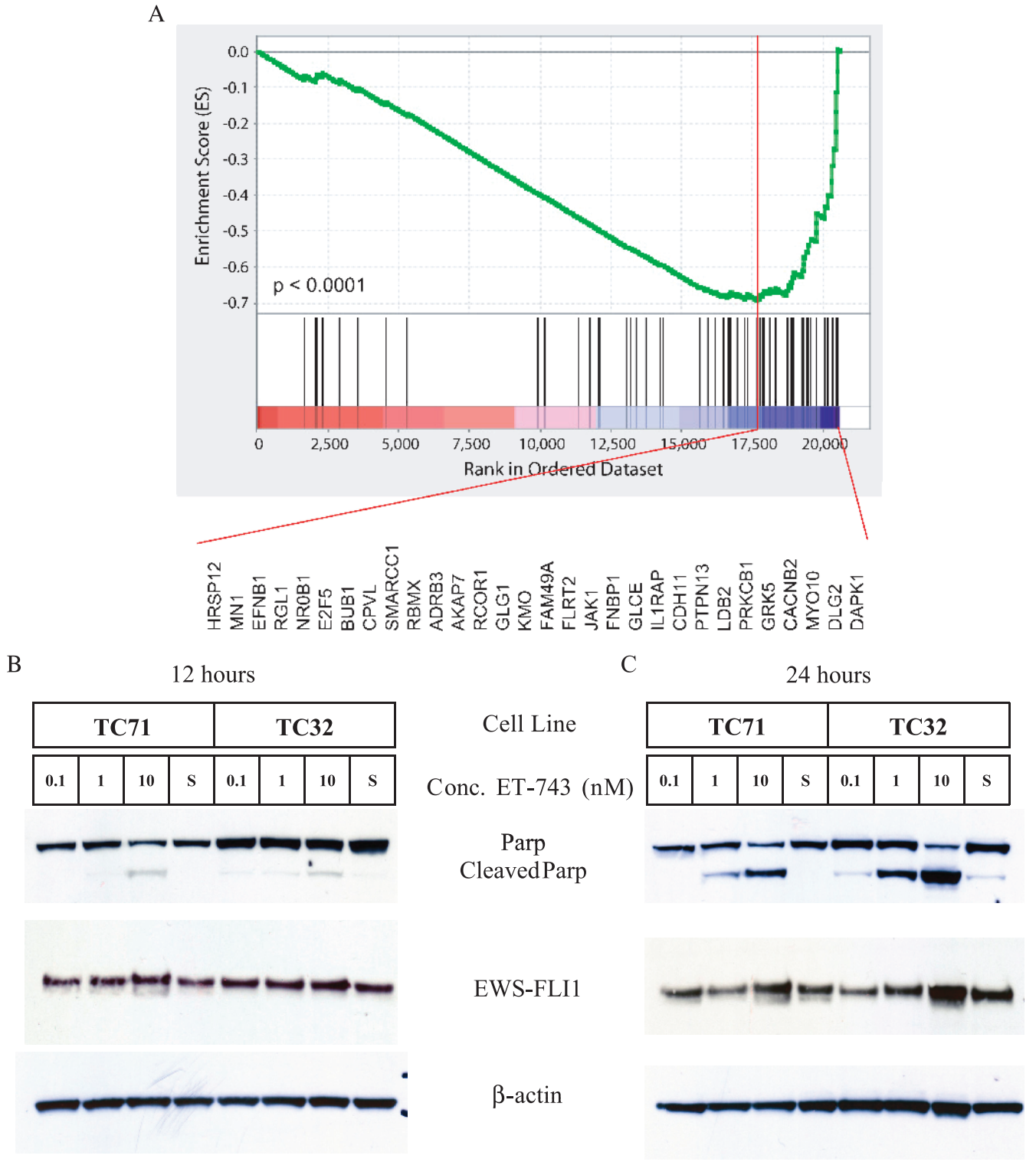


Figure 2. ET-743 decreases the expression of the downstream targets of EWS-FLI1 without changing the expression of the EWS-FLI1 transcription factor. (A) Enrichment plot showing an EWS-FLI1 activated gene set obtained from a published meta-analysis [5]. ET-743 reverses EWS-FLI1–activated genes in a statistically significant manner ($P < .001$). The curve shows the running sum of enrichment score (ES) for ranked proteins. The vertical line specifies the maximum ES score. The genes listed under the plot are the leading edge subset. (B) Western blot demonstrates no change in expression of EWS-FLI1 in two different ESFT sarcoma cell lines TC32 and TC71 when treated with ET-743 for 12 and 24 hours. There is minimal apoptosis observed at 12 hours as evidence by minimal cleaved PARP. By 24 hours, significant apoptosis has occurred at both 10 and 1 nM. β-Actin confirms equal loading of protein lysate (C).

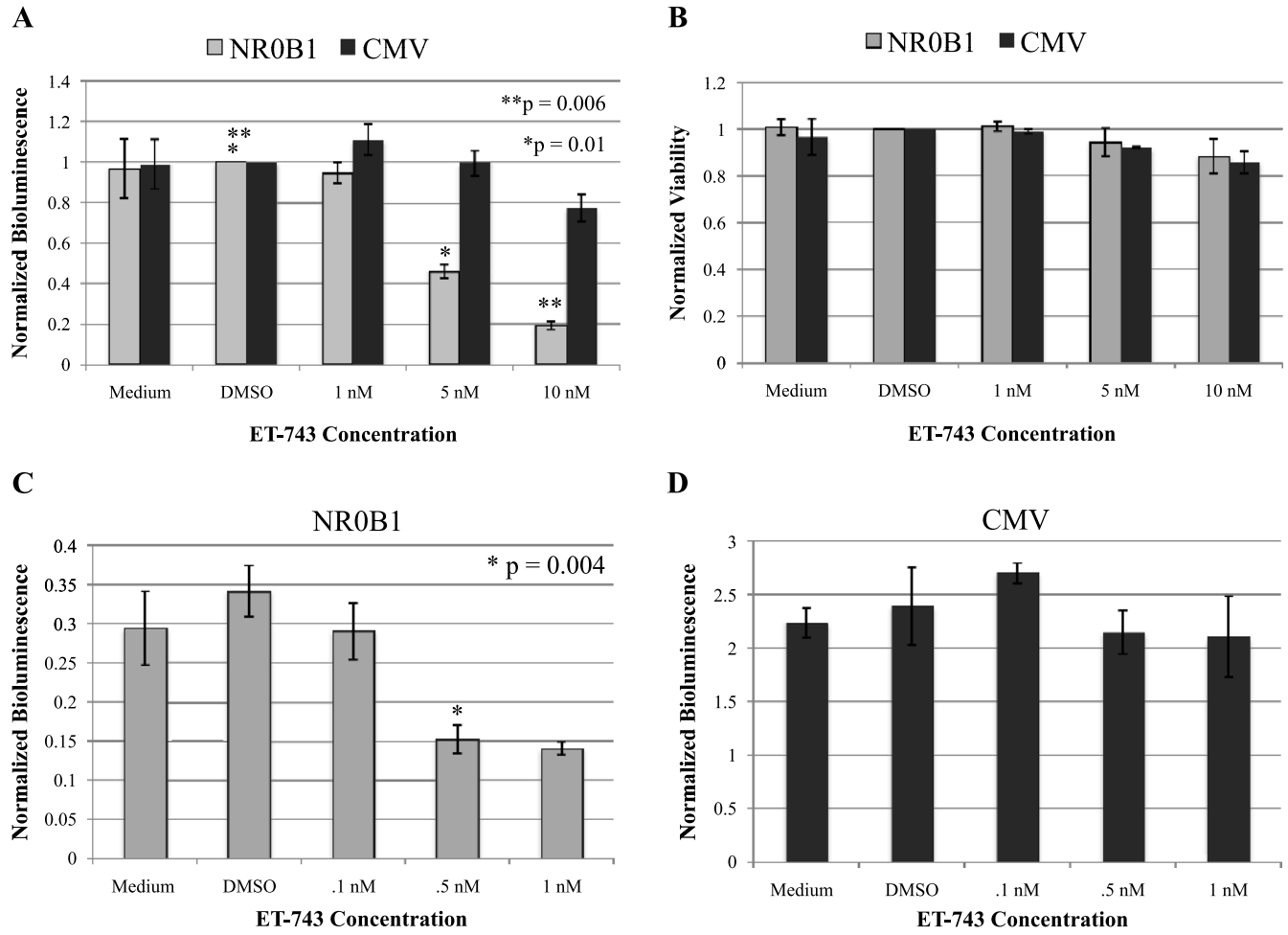


Figure 3. ET-743 selectively modulates the expression of the EWS-FLI1 downstream target promoter NR0B1 luciferase construct. (A) Luciferase activity is markedly decreased with 10 nM ET-743 treatment at 12 hours in cells that contain the NR0B1 promoter construct ($P = .006$). A dose-dependent effect is observed with almost 50% inhibition of luciferase in the 5-nM sample ($P = .01$). No effect is observed at the identical 5-nM concentration and time with the CMV promoter construct. Data are normalized to the solvent control. (B) There is a minimal effect on cell viability at the time and concentration tested. (C, D) The inhibition of luciferase in the NR0B1 promoter luciferase construct ($P = .004$) but not the CMV is observed at similar concentrations as the IC_{50} after a longer 24-hour exposure. In this case, the data are expressed normalized to the cell number to account for the nonspecific decrease in luciferase expression caused by cell death.

UVC treatment of fibroblasts taken from patients with xeroderma pigmentosa, trichothiodystrophy, or Cockayne syndrome [39] were found to be statistically significant. Interestingly, a similar comparative analysis done of a Cockayne syndrome gene set identified considerable overlap with ET-743 gene signatures [40]. We also identified gene sets that suggest a relationship with differentiation (HSC_Mature_adult [41]) and chromatin remodeling (Table 1).

In addition, there was overlap in our expression data with a curated list of genes that reflect ET-743–sensitive sarcoma cell lines that included one ESFT and four myxoid liposarcoma cell lines (ET743_Sarcoma_DN) [42]. It is notable that a number of curated gene sets that represent data generated by treating other cell types with ET-743 were not identified including a set of genes from ET-743–resistant sarcoma cell lines as well as chondrosarcoma cells and ovarian carcinoma cells that were profiled after selecting for resistance to ET-743.

Discussion

ET-743 is a natural product isolated from the marine sea squirt *E. turbinata*. This compound exhibits a strong cytotoxic effect in a variety of cancer cell types of both epithelial and mesenchymal origin. The broad cytotoxic profile likely stems from ET-743's primary mechanism of action of alkylating the N2 position of guanine at selective sequences [24,25], which in turn poisons TCR [29–31] and inhibits the transcription of specific gene sets [23,27,28].

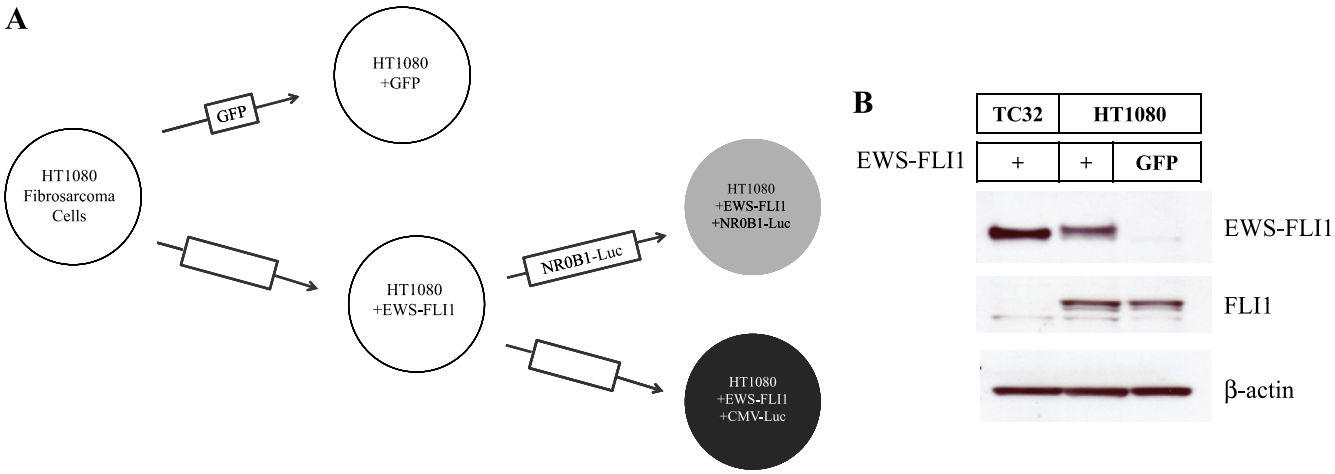
Despite the broad range of cytotoxicity, the clinical trials of ET-743 have revealed some preferential activity against sarcomas most notably myxoid liposarcoma, a tumor characterized by the FUS-CHOP oncogenic transcription factor [16–22]. Recently, it was shown that ET-743 directly interferes with the FUS-CHOP–mediated block in differentiation in myxoid liposarcoma [23]. It has been suggested that release from this differentiation block explains the selective sensitivity of this tumor type to ET-743 in the clinic. However, it is not known what, if any, other tumors bearing fusion transcription factors might be particularly sensitive to the drug.

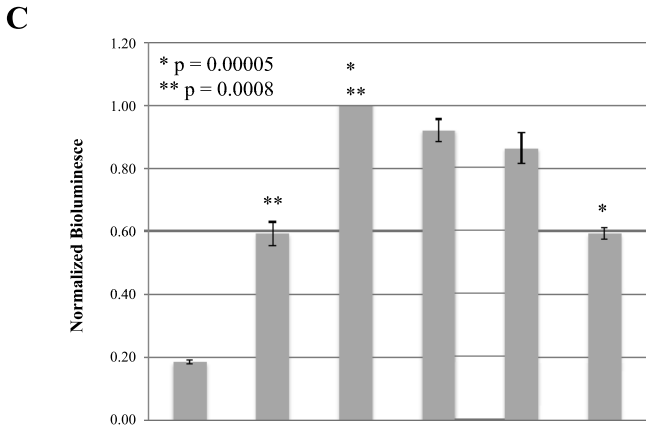
In this report, we demonstrate that the sensitivity of ESFT cells to ET-743 is associated with the modulation of EWS-FLI1 activity. We show that ESFT cells are more sensitive than other pediatric sarcoma cell lines and that this sensitivity correlates with the modulation of an

EWS-FLI1 regulated promoter. In addition, this effect can be recapitulated in a different cellular context by transducing a fibrosarcoma cell line with EWS-FLI1 leading to a strong induction of the NR0B1 promoter that is completely blocked with ET-743 treatment.

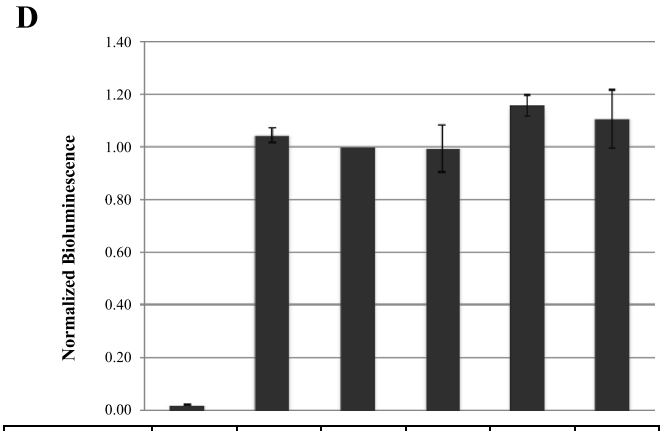
Finally, we show that the level of expression of EWS-FLI1 does not change, but the expression of a gene signature of induced downstream targets of EWS-FLI1 is reversed and leads in part to the subsequent induction of apoptosis. The reversal of the EWS-FLI1 gene signature would not be expected for a general transcription inhibitor, and instead, this suggests a relationship between this gene signature and the cellular response to drug treatment. In addition, the luciferase data that use a CMV-driven control further argue against a general mechanism of global transcription inhibition.

It is not clear if the gene signature reversal demonstrated by GSEA stems from a direct block in DNA binding of EWS-FLI1 similar to what has been reported for FUS-CHOP [23] or some other indirect mechanism. The luciferase reporter studies suggest that the effect occurs at the level of the downstream target promoter. This mechanism is plausible given the overlapping binding domains of EWS-FLI1 and ET-743. The original report that characterized the DNA binding domain of FLI1 used an epitope tagging strategy coupled with *in vitro* selection to determine the optimal FLI1 binding sequence. The report identifies a consensus FLI1 binding sequence of ACCGGAAG/aT/c and shows a greater sequence dependence on nucleotides 5′ to the GGAA core than other *ets* transcription factors. In addition, changing the nucleotide immediately 5′ to the core from a C to an A blocks





GFP	+	+	0	0	0	0
NR0B1-Luc	0	+	+	+	+	+
EWS-FLI1	0	0	+	+	+	+
ET-743 (nM)	0	0	S	1	5	10



GFP	+	+	0	0	0	0
CMV-Luc	0	+	+	+	+	+
EWS-FLI1	0	0	+	+	+	+
ET-743 (nM)	0	0	S	1	5	10

Figure 4. EWS-FLI1 activates the NR0B1 promoter with forced expression of EWS-FLI1 in the HT1080 cell line that normally lacks the fusion. ET-743 interferes with the activation of NR0B1 in this HT1080 cell line that expresses EWS-FLI1. (A) Schematic showing HT1080 transduction experiment. (B) Western blot confirming the presence of the EWS-FLI1 oncogene in the HT1080 cells. The Western blot also shows TC32 lysate as a control. EWS-FLI1 in the HT1080 cells is slightly shifted because it contains an HA-tag. (C) HT1080 cells that contain EWS-FLI1 activate the NR0B1 promoter by 40% more than baseline with forced expression of EWS-FLI1 ($P = .0008$). However, treatment of the cells with 10 nM ET-743 returns this activation to baseline ($P = .00005$). (D) The CMV-driven luciferase shows high-level luciferase activity that does not change either with the introduction of EWS-FLI1 or with ET-743 treatment. All luciferase data are expressed as normalized to the solvent control.

Table 1. Top 11 Gene Sets That Showed the Most Overlap with the Gene Signature Generated by Treating TC32 and TC71 Cells with ET-743.

Name	Size	NOM, *P*	FDR, *q*
UV damage			
UVB_NHEK1_DN	270	—	—
UV damage and deficient TC-NER			
UVC_XPCS_ALL_DN	478	—	—
UVC_TTD_ALL_DN	358	—	—
Activation of apoptosis			
APOPTOSIS	67	—	0.0247949
APOPTOSIS_GENMAPP	42	.006079027	0.033063594
ET-743 sarcoma			
ET743_SARCOMA_DN	269	—	0.027059516
Differentiation			
HSC_MATURE_ADULT	331	—	0.023825139
Chromatin remodeling			
Above XPCS/TTD			
HDACI_COLON_SUL_DN	215	—	0.020208418
BAF57_BT549_UP	238	—	1.98*e*–04
BRCA_BRCA1_NEG	154	—	0.021794723

Data shows the name of the original experiment including the size of the gene set, the associated *P* value, and the FDR. Gene sets associated with cell or DNA damage, activation of apoptosis, differentiation, and chromatin remodeling were identified. In addition, a previous experiment that used expression profiling in ET-743–sensitive sarcoma cell lines showed significant overlap of expression changes.

FLI1 binding [3]. This is significant because the preferred binding domain of ET-743 is CGG, whereas AGG is disfavored. Therefore, a CGGAA sequence would exactly overlap the preferred binding sequence of ET-743, interrupt EWS-FLI1 binding, and account for the results presented in this report.

Consistent with this notion is the finding that the ESFT cell line 5838 containing the EWS-ERG translocation is less sensitive to ET-743 than those ESFT cell lines that have an EWS-FLI1 translocation. Recently, an ERG consensus sequence was identified to be (A/G)(G/C)AGGAA(A/G) and was not activated by FLI1 suggesting some but not complete overlap of these *ets* transcription factor family members activities [43]. In addition, The ERG *ets* transcription factor binding domain features an A immediately 5′ to the GGAA core and therefore does not have a preferred ET-743 binding site overlapping its binding domain [43]. Work continues to definitively demonstrate a change in the chromatin binding of EWS-FLI1 in the presence of ET-743. To date, our chromatin immunoprecipitation experiments have not been of sufficient quality to definitively demonstrate a mechanism, and we continue to pursue alternative strategies aimed at determining the effect of ET-743 on EWS-FLI1 binding to native chromatin.

Nevertheless, the similarities of myxoid liposarcoma and ESFT transcription factor dynamics most likely contribute to the sensitivity to ET-743 in these two tumor types. Both cells have a dependence on their transcriptional program for maintenance of the oncogenic phenotype. In addition, EWS-FLI1 and FUS-CHOP share overlapping DNA binding domains with ET-743, although the sequences are different. EWS-FLI1 and ET-743 share the CGG binding domains in the coding strand, whereas the complementary strand of the CCAAT FUS-CHOP binding domain contains an ET-743 TGG binding sequence. Likely the overlapping binding domains coupled with the inherent dependence of these two cell types on the corresponding transcriptional signatures is additive with the well described mechanisms of ET-743 and explains the heightened sensitivity of these cells to ET-743.

Finally, an analysis of the GSEA enrichment data provides further evidence that the effects of EWS-FLI1 inhibition are additive with the other well characterized mechanisms in ESFT cells. Most notably, gene sets generated by UVB or UVC treatment of fibroblasts taken from patients with xeroderma pigmentosa, trichothiodystrophy, or Cockayne syndrome [39] were identified as similar to ET-743 treatment of ESFT cells. Patients with xeroderma pigmentosa, trichothiodystrophy, and Cockayne syndrome are born with a defect in TCR. Therefore, our finding that treatment of ESFT cells with ET-743 leads to global gene changes similar to UV damage in patients with defective TCR suggests that this mechanism of generating UV-like damage and poisoning TCR is active in ESFT cells.

Finally, one would hypothesize that the reversal of activity of an oncogenic transcription factor would lead to chromatin remodeling and release from a block in differentiation similar to what has been described for FUS-CHOP and myxoid liposarcoma [23]. Work continues to investigate whether this is the case in ESFT cells; however, our GSEA identified gene sets that were statistically similar to ET-743 treatment of ESFT cells and involved chromatin remodeling and a release of a block in differentiation in other cell types.

In summary, ESFTs have been shown to be particularly sensitive to ET-743 *in vitro*, and there are some data supporting its activity in the clinic [20]. We hypothesize that the reversal of the genetic program created by the oncogenic transcription factor likely provides an additional mechanism of cytotoxicity that is additive to the known mechanisms of cytotoxicity of ET-743 in ESFT. It is not clear if this effect is based on a direct block of EWS-FLI1 binding or an indirect mechanism. A detailed genome-wide chromatin structure study would be required to answer this question. Nevertheless, the demonstrated modulation of activity of EWS-FLI1 coupled with early clinical trial results suggests that further development of ET-743 in ESFT is warranted. In addition, a heightened sensitivity for translocation-positive tumors based on the blockade of their associated oncogenic transcription factor with ET-743 treatment is suggested, and a close examination of the effects of ET-743 on a variety of oncogenic transcription factors may identify other important targets.

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Table W1. Suppressed Gene Sets That Overlap with the Genes That Suppressed with ET-743 Treatment in ESFT Cells.

Name	Size	NOM, *P*	FDR, *q*
UVC_XPCS_ALL_DN	478	<.001	0.0000
UVC_XPCS_8HR_DN	408	<.001	0.0000
DIAB_NEPH_DN	374	<.001	0.0155
UVC_TTD_ALL_DN	358	<.001	0.0000
HSC_MATURE_ADULT	331	<.001	0.0238
UVC_TTD_4HR_DN	297	<.001	0.0000
UVB_NHEK1_DN	270	<.001	0.0000
ET743_SARCOMA_DN	269	<.001	0.0271
UVC_XPCS_4HR_DN	242	<.001	0.0000
BAF57_BT549_UP	238	<.001	0.0002
HDACI_COLON_SUL_DN	215	<.001	0.0202
VENTRICLES_UP	205	<.001	0.0258
HSA04810_REGULATION_OF_ACTIN_CYTOSKELETON	198	<.001	0.0315
HSA04510_FOCAL_ADHESION	194	<.001	0.0100
GH_GHRHR_KO_24HRS_DN	172	<.001	0.0050
UVC_TTD_8HR_DN	165	<.001	0.0000
OLD_FIBRO_DN	158	<.001	0.0202
BRCA_BRCA1_NEG	154	<.001	0.0218
UVC_TTD-XPCS_COMMON_DN	144	<.001	0.0000
BRENTANI_PROTEIN_MODIFICATION	143	<.001	0.0104
GH_GHRHR_KO_24HRS_UP	142	<.001	0.0199
CALCIUM_REGULATION_IN_CARDIAC_CELLS	140	<.001	0.0318
HSA04530_TIGHT_JUNCTION	132	<.001	0.0063
UVB_NHEK1_C6	130	<.001	0.0000
HSA04360_AXON_GUIDANCE	128	<.001	0.0038
GH_AUTOCRINE_DN	122	<.001	0.0039
HSA01030_GLYCAN_STRUCTURES_BIOSYNTHESIS_1	111	<.001	0.0362
G_PROTEIN_SIGNALING	90	<.001	0.0070
PHOSPHATIDYLINOSITOL_SIGNALING_SYSTEM	88	<.001	0.0002
BRCA1KO_MEF_DN	79	<.001	0.0209
ST_INTEGRIN_SIGNALING_PATHWAY	78	<.001	0.0054
CREB_BRAIN_8WKS_UP	75	<.001	0.0042
HSA04520_ADHERENS_JUNCTION	75	<.001	0.0157
HSA04664_FC_EPSILON_RI_SIGNALING_PATHWAY	74	<.001	0.0283
CARIES_PULP_DN	70	<.001	0.0299
HSA04720_LONG_TERM_POTENTIATION	69	<.001	0.0052
UVB_NHEK3_C8	66	<.001	0.0002
UVC_LOW_ALL_DN	58	<.001	0.0000
OLDONLY_FIBRO_DN	53	<.001	0.0092
NFATPATHWAY	52	<.001	0.0006
HDACI_COLON_CUR_DN	49	<.001	0.0002
ET743PT650_COLONCA_DN	44	<.001	0.0000
NOVA2_KO_SPLICING	42	<.001	0.0019
CMV_HCMV_TIMECOURSE_14HRS_DN	41	<.001	0.0089
BIOPEPTIDESPATHWAY	38	<.001	0.0002
DFOSB_BRAIN_2WKS_UP	38	<.001	0.0010
UVB_NHEK3_C5	35	<.001	0.0000
AT1RPATHWAY	34	<.001	0.0068
GLYCOGEN_METABOLISM	34	<.001	0.0159
HSA00970_AMINOACYL_TRNA_BIOSYNTHESIS	31	<.001	0.0071
HDACPATHWAY	30	<.001	0.0002
CREBPATHWAY	27	<.001	0.0007
HDACI_COLON_CUR24HRS_DN	25	<.001	0.0069

The microarray data generated by treating TC32 and TC71 with ET-743 was compared with more than 1892 curated gene sets using GSEA [36]. All had $P < .001$ and FDR < 0.05.
The source of the data can be found on the Web site (http://www.broad.mit.edu/gsea/) and is referenced in the text where appropriate.
Shaded data indicate data sets discussed in the text.
Size indicates the number of genes in the gene set that shows overlap with the ones in the expression data set.

Table W2. Induced Gene Sets That Showed Significant Overlap.

Name	Size	NOM, *P*	FDR, *q*
CMV_HCMV_TIMECOURSE_ALL_UP	464	<.001	0.0393
REOVIRUS_HEK293_UP	236	<.001	0.0053
CARIES_PULP_UP	205	<.001	0.0085
UVB_NHEK1_UP	173	<.001	0.0006
CMV-UV_HCMV_6HRS_UP	120	<.001	0.0054
BLEO_HUMAN_LYMPH_HIGH_24HRS_UP	92	<.001	0.0008
LVAD_HEARTFAILURE_UP	89	<.001	0.0025
UVB_NHEK3_C0	82	<.001	0.0017
HYPOXIA_REVIEW	81	<.001	0.0125
UVC_TTD_ALL_UP	76	<.001	0.0000
DNMT1_KO_UP	72	<.001	0.0045
APOPTOSIS	67	<.001	0.0248
BRENTANI_TRANSCRIPTION_FACTORS	64	<.001	0.0236
UVC_XPCS_ALL_UP	61	<.001	0.0000
UVC_XPCS_8HR_UP	58	<.001	0.0000
UVC_TTD_4HR_UP	58	<.001	0.0020
ET743_HELA_UP	56	<.001	0.0007
CMV_HCMV_6HRS_DN	55	<.001	0.0000
CMV_HCMV_TIMECOURSE_6HRS_DN	52	<.001	0.0007
TPA_SENS_LATE_UP	52	<.001	0.0394
CMV_HCMV_TIMECOURSE_14HRS_UP	45	<.001	0.0000
ZUCCHI_EPITHELIAL_DN	43	<.001	0.0008
TNFALPHA_30MIN_UP	42	<.001	0.0065
EGF_HDMEC_UP	42	<.001	0.0066
ADIP_DIFF_CLUSTER2	40	<.001	0.0002
HYPOXIA_REG_UP	38	<.001	0.0000
DSRNA_UP	38	<.001	0.0002
IFN_GAMMA_UP	38	<.001	0.0014
STRESS_GENOTOXIC_SPECIFIC_UP	34	<.001	0.0045
ST_TUMOR_NECROSIS_FACTOR_PATHWAY	29	<.001	0.0065
OXSTRESS_BREASTCA_UP	29	<.001	0.0071
DAC_BLADDER_UP	28	<.001	0.0046
CMV_UV-CMV_COMMON_HCMV_6HRS_DN	27	<.001	0.0064
IFNALPHA_NL_UP	27	<.001	0.0084
CMV_HCMV_6HRS_UP	25	<.001	0.0010
AD12_ANY_DN	25	<.001	0.0034
IDX_TSA_UP_CLUSTER1	25	<.001	0.0048

The microarray data generated by treating TC32 and TC71 with ET-743 was compared with more than 1892 curated gene sets using GSEA [36]. All had $P < .001$ and FDR < 0.05.
The source of the data can be found on the Web site (http://www.broad.mit.edu/gsea/) and is referenced in the text where appropriate.
Size indicates the number of genes in the gene set that shows overlap with the ones in the expression data set.